Supplementary Table S1

Strain	Relevant genotype
YMV20	*MAT***a** *MUS81-3HA::HIS3MX6*
YMV33	*MAT***a** P_{ADH1}*-3HA-MMS4::HIS3MX6*
YMG1	*MAT***a** *cdc28Δ::cdc28-td::URA3 ubr1Δ::*$P_{GAL1,10}$*-Myc-UBR1::HIS3* P_{ADH1}*-3HA-MMS4::natNT2*
YMG2	*MAT***a** *ubr1Δ::*$P_{GAL1,10}$*-Myc-UBR1::URA3* P_{ADH1}*-3HA-MMS4::natNT2*
YMG3	*MAT***a** *ade1 ade2 tyr1 his7 lys2 gal1-1 cdc5-1* P_{ADH1}*-3HA-MMS4::natNT2*
YSG22	*MAT***a** *MMS4-TAP::kanMX4*
YMV48	*MAT***a** *mms4Δ::URA3*
YJT161	*MAT***a** *yen1Δ::LEU2*
YMG7	*MAT***a** *sgs1Δ::kanMX4*
YMG9	*MAT***a** *sgs1Δ::kanMX4 yen1Δ::LEU2*
YMG10	*MAT***a** *mms4Δ::URA3 yen1Δ::LEU2*
YMG11	*MAT***a** *mms4Δ::URA3::*P_{ADH1}*-3HA-mms4-np (S55A; S56A; S184A; S201A; S221A; S222A; S301A; T302A; S403A)::HIS3*
YMG12	*MAT***a** *nat-NT2 mms4Δ::URA3::* P_{ADH1}*-3HA-mms4-np (S55A; S56A; S184A; S201A; S221A; S222A; S301A; T302A; S403A)::HIS3 sgs1Δ::kanMX4*
YMG13	*MAT***a** *nat-NT2 mms4Δ::URA3::pADH1-3HA-mms4-np (S55A; S56A; S184A; S201A; S221A; S222A; S301A; T302A; S403A)::HIS3 yen1Δ::LEU2*
YMG14	*MAT***a** *nat-NT2 mms4Δ::URA3::pADH1-3HA-mms4-np (S55A; S56A; S184A; S201A; S221A; S222A; S301A; T302A; S403A)::HIS3 sgs1Δ::kanMX yen1Δ::LEU2*
YMG21	*MAT***a** *sgs1Δ::kanMX4* P_{ADH1}*-3HA-MMS4::natNT2*

Table S1. *S. cerevisiae* strains used in this study. All the strains were constructed for this work by standard techniques, using the pML (47) or pYN (48) plasmid series as templates for PCR. All the strains were based on W303 (*ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100),* except YMG3, based on A364A. The *cdc28-td* and *cdc5-1* mutants were kindly provided by J.F.X. Diffley (Cancer Research UK).

Supplementary Table S2

Oligonucleotide name	Sequence
3'FL-1	5'-GATCACAGTGAGTACCGG
3'FL-2	5'-ACTGGCCGTCGTTTGTACTCACTGTGATC
3'FL-3	5'-AACGACGGCCAGT
RF-1	5'-GACGCTGCCGAATTCTGGCGTTAGGAGATACCGA TAAGCTTCGGCTTAAG
RF-2	5'-ATCGATGTCTCTAGACAGCACGAGCCCTAACGCC AGAATTCGGCAGCGTC
RF-3	5'-CTTAAGCCGAAGCTTATCGGTATCT
RF-4	5'-GCTCGTGCTGTCTAGAGACATCGAT
X0-1	5'-ACGCTGCCGAATTCTACCAGTGCCTTGCTAGGAC ATCTTTGCCCACCTGCAGGTTCACCC
X0-2A	5'-GGGTGAACCTGCAGGTGGGCAAAGATGTCC
X0-2B	5'-ATCTGTTGTAATCGTCAAGCTTTATGCCGT
X0-3	5'-ACGGCATAAAGCTTGACGATTACAACAGATCATG GAGCTGTCTAGAGGATCCGACTATCG
X0-4	5'-CGATAGTCGGATCCTCTAGACAGCTCCATGTAGC AAGGCACTGGTAGAATTCGGCAGCGT

Table S2. Oligonucleotides used to make the DNA substrates for the nuclease activity assays. 3'FL-1 to -3 were used to make a synthetic 3'-flap structure. RF-1 to -4 were based on (13) and used to make a model replication fork. X0-1 to -4 were based on (60) and used to make a nicked Holliday junction.

Figure S1. Amino acid sequence of wild-type Mms4 (A) and mutant Mms4-np (B). The CDK phosphorylation consensus sites (T/S-P-X-K/R and T/S-P) and the potential docking sites for Polo-like kinases (S-pS/pT-P/X) are in boxes. Highlighted in bold (A) are the serine and threonine residues that were mutated to alanines (B).

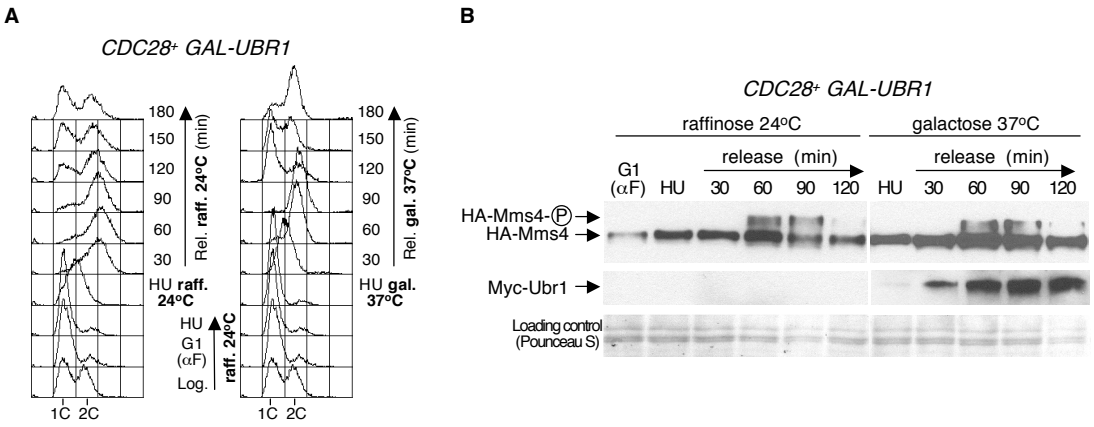

Figure S2. Cell cycle-dependent phosphorylation of Mms4 in a *CDC28+ GAL-UBR1* strain. The *CDC28+ P*$_{GAL1\text{-}10}$*-UBR1 HA-MMS4* cells were blocked in G1 with α factor and released in fresh medium with HU. The culture was then split in two: one half was incubated in medium with raffinose (*GAL1-10* promoter "OFF") at 24°C; in the other half, the expression of *UBR1* was induced upon addition of galactose after shifting the cells to 37°C. In both cases, the cells were released from the HU arrest. The cells were collected for analysis at the indicated time points. (A) The DNA content during the course of the experiment was determined by flow cytometry. (B) Immunoblot analysis of Mms4 and Ubr1 during the course of the experiment. Phosphorylated Mms4 is indicated as 'Mms4-P'.

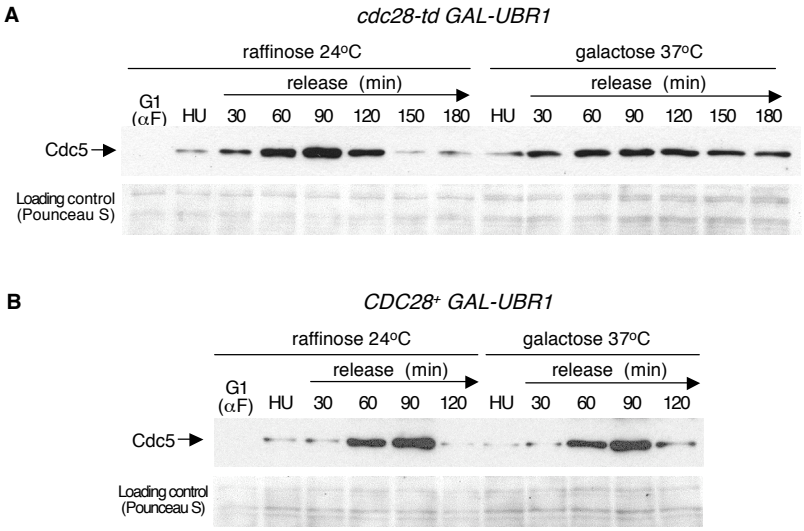

Figure S3. Cell cycle-regulated expression of Cdc5 in a *cdc28-td* mutant. Immunoblot analysis of Cdc5 during the course of the experiments. (A) Expression of Cdc5 in a *cdc28-td GAL-UBR1* strain. Samples are from the experiment described in Figure 2A. The peak of expression of Cdc5 at the permissive temperature coincides with the time of Mms4 phosphorylation (Figure 2A). At the non-permissive temperature, 37ºC, the peak of expression of Cdc5 occurs approximately at the same time than at 24ºC, and the protein levels are maintained due to the block in G2/M (Figure 2A). (B) Expression of Cdc5 in the *CDC28+ GAL-UBR1* strain used as a control. Samples are from the experiment described in Supplementary Figure S2. The peak of expression of Cdc5 coincides with the time of Mms4 phosphorylation (Figure S2). Pounceau S stained membranes were used as loading controls.

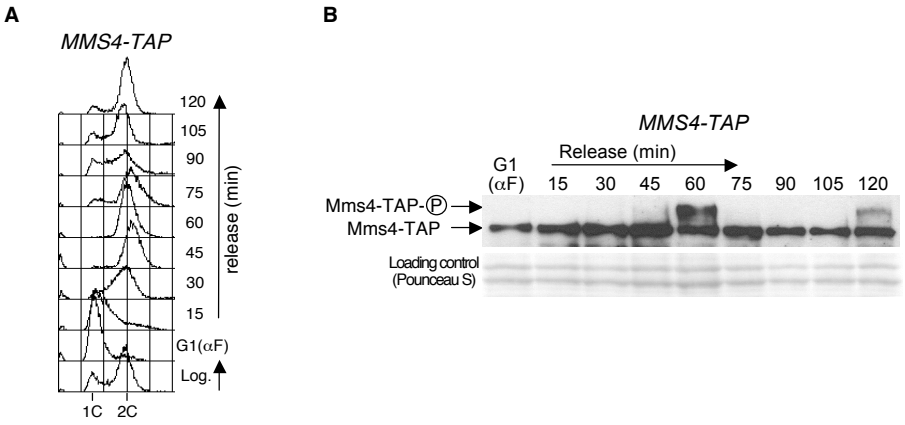

Figure S4. Cel cycle-dependent modification of Mms4-TAP. The *MMS4-TAP* cells were blocked in G1 with α factor and released in fresh medium. The cells were collected at the indicated time points. (A) The DNA content throughout the experiment was determined by flow cytometry. (B) Immunoblot analysis of Mms4 during the course of the experiment. The phosphorylated form of Mms4 is indicated as Mms4-P.

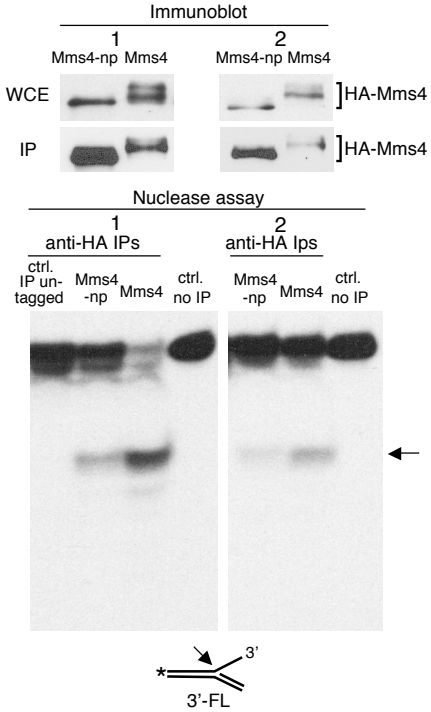

Figure S5. Reduced nuclease activity in the phosphorylation-defective mutant *mms4-np*. The amount of the product resulting from nucleolytic cleavage by Mus81-Mms4 correlates with the amount of immunoaffinity purified Mms4 (wild type or mutant). The extracts were prepared from *HA-mms4-np* and *HA-MMS4* cells blocked in G2/M with nocodazole. The extracts were obtained in parallel experiments from $7.5x10^8$ cells (1) or from $3.75x10^8$ cells (2). The experiment in (1) is the same than that in Figure 4 and is shown here for comparison with (2). The phosphorylation of wild-type Mms4 and mutant Mms4-np in the whole cell extract (WCE), as well as the yield of the protein immunoprecipitation (IP) were monitored by immunoblot (upper panels). 2% of the total amount of immunoprecipitated protein used for the nuclease assays was loaded in each case. The nuclease activity was assayed using a ^{32}P-labelled 3'-flap as a substrate (lower panel). An arrow indicates the labelled product resulting from the nucleolytic cleavage. The controls were a nuclease assay using an immunoprecipitated extract from untagged cells blocked in G2/M and reactions in the absence of extract.